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(54) Title: AFFINITY SELECTION OF LIGANDS BY MASS SPECTROSCOPY

(57) Abstract

Compounds are quickly selected from a combinatorial library by contacting the library with a target (e.g., receptor), separating non-binding compounds from compound-target complexes, and analyzing the complexes or eluted compound by mass spectroscopy. SAR information is obtained by performing this selection at two or more different ratios of compound to target.

Affinity Selection of Ligands by Mass Spectroscopy

Description

5   ## Technical Field

This invention relates to the fields of drug discovery and mass spectroscopy. More particularly, the invention relates to the use of mass spectroscopy to identify ligands that bind a selected receptor from a pool of similar ligands.

10   ## Background of the Invention

Geysen, EP 198855, disclosed a method for the simultaneous synthesis of a large number of different peptides. Basically, this method involves the synthesis of peptides on a solid polymeric surface, such as polyethylene, which may be molded into the shape of a rod or pin. In a preferred embodiment of the method, these rods or pins are positioned in a

15   holder so that they form a 12 by 8 matrix, with the rods or pins being positioned so that the spacing corresponds to that of the wells of microtiter plates which are widely used for ELISA (enzyme-linked immunosorbent assay) tests.

Huebner et al., US 5,182,366 (incorporated herein by reference) disclosed a method for preparing large mixtures of peptides on solid phase resins in equimolar ratios. This

20   enables one to quickly search for compounds that bind to or react with a ligand by contacting the ligand (e.g., a bound receptor) with a set of peptide mixtures and noting which members of the set bind or react. Typically, the sets are prepared by specifying a known amino acid at one or two positions of an oligopeptide and providing mixtures of amino acids at the other positions. Thus, one peptide mixture might consist of a pool of

25   hexapeptides of the formula Gly-Gly-X_1-X_2-X_3-X_4, where each X indicates that all amino acids are found at that position. The next peptide mixture would be Gly-Ala-X_1-X_2-X_3-X_4, followed by Gly-Cys-X_1-X_2-X_3-X_4, and so forth. The set consisting of all of these mixtures is termed a "library." A library is screened by testing each individual mixture and noting which mixtures produce a positive response. In some formats, the mixtures may be

30   screened simultaneously. The positive mixtures are then resynthesized with additional positions specified. Thus, for example, if the mixture containing Phe-Tyr-X_1-X_2-X_3-X_4 was

positive, the next mixture synthesized might be Phe-Tyr-Gly-X_2-X_3-X_4. This process

(called "deconvolution") is reiterated until individual peptides are synthesized and tested.

Bartlett *et al.*, WO91/19735, and Zuckermann *et al.*, WO94/06451 disclosed a

method for extending combinatorial library synthesis to compounds other than peptides.

5   Bartlett and Zuckermann disclosed modular compounds based on N-substituted

polyamides, poly-carbamates, and other backbones, which permits one to research non-

peptide compounds. These libraries are also analyzed by deconvolution.

Disclosure of the Invention

10   We have now invented a method for directly determining the identity of binding

ligands by mass spectroscopy, eliminating the need to deconvolute pools of compounds.

This permits one analyze combinatorial library results directly, without resynthesizing the

mixtures.

Another aspect of the invention is a method for determining the relative affinities of

15   similar active compounds present in a mixture, by selecting compounds at two or more

different compound:target ratios and comparing the spectra.

Modes of Carrying Out The Invention

20   A. Definitions

The term "target" refers to any compound, protein, receptor, or the like, for which

a binding or reacting compound is sought. For example, if a library is screened for

compounds which bind to the endothelin receptor, the endothelin receptor is the target.

The target may be soluble, or immobilized, *e.g.*, on a solid phase or cell surface.

25   The term "compound" refers to a chemical entity which may be analyzed by mass

spectroscopy, and which may have biological activity. Compounds within the scope of this

invention preferably have a molecular weight of about 100 to about 1,000 AU, more

preferably about 200-900 AU. The term "similar compounds" refers to a set of compounds

that are made by substantially the same synthetic method or share certain properties. For

30   example, a mixture of peptides constitutes a set of similar compounds within the scope of

this definition. A set of peptoids based on a common backbone is a set of similar

compounds, regardless of the difference in polarity, hydrophobicity, pK, or other properties.

The term "mixture of similar compounds" refers to a solution or suspension of similar compounds (as defined above) having at least 10 distinct compounds, more preferably at least 100 different compounds, still more preferably at least 1,000 different compounds.

B. General Method

Affinity selection is conducted using mixtures of compounds, typically resulting from a combinatorial library. The compounds are preferably soluble, rather than bound to a solid phase, and preferably have a molecular weight between about 200 and about 700 Daltons.. The compounds may be prepared by any method, preferably as described by Bartlett *et al.*, WO91/19735, or Zuckermann *et al.*, WO94/06451, both incorporated herein by reference.

The target may be either bound or soluble, but for kinetic reasons is preferably soluble. The target is typically provided using common techniques of molecular biology. Immobilized targets are provided, for example by expressing a cloned receptor on the surface of a suitable host cell (*e.g.*, a transfected CHO cell or recombinant baculovirus-infected Sf9 cell), or by expressing the receptor in soluble form (*e.g.*, by truncating the receptor so as to exclude a transmembrane anchor domain) and immobilizing it on a suitable surface (for example, by non-specific affinity to nylon or polystyrene assay plates, or by specific binding using antibodies or biotin-avidin coupling systems).

The target and compounds (or library) are brought into contact, either in solution phase or with one component bound to a solid phase, allowed to react or bind, and the non-binding compounds separated from the binding compounds (or compound-target complexes). If the compounds or target are bound to a solid phase, this separation is easily accomplished by washing the solid phase support. If the assay is performed in solution, the separation may be accomplished by means of a sizing column or affinity capture of the target. Sizing columns are generally useful because all compounds in a given library are likely to be of similar size, and are likely to be much smaller than the target or compound-target complex. Affinity capture may be employed, for example, by providing the target

with a "tag" or ligand that binds to an antibody or binding partner (*e.g.*, avidin/biotin). It is presently preferred to separate non-bound compounds from compound-target complexes by means of rapid size exclusion chromatography HPLC (SEC-HPLC).

Once separated, the compounds are either eluted from the targets, or are submitted directly for mass spec (MS) analysis. Elution may be accomplished by dilution, pH, competition with a specific ligand, and the like. Some MS ionization methods, such as electrospray, APCI, laser desorption, and electron impact, are sufficient to dissociate the compound from the target, and thus may be used directly, without prior elution. Thus, an SEC-HPLC column may be coupled directly to the input of an ES MS for formation of a rapid throughput instrument. With knowledge of the potential compounds present (*i.e.*, the compounds present in the initial mixture), one can typically identify most or all binding compounds selected from the mixture using an MS detector having a resolution of about 1 A.U.

The exact parameters used (*e.g.*, solvents, temperatures, pH, and the like) will necessarily depend on the compounds and target selected, due to the broad, general nature of the method of the invention and the diversity of possible compounds and targets. However, such parameters may be determined using only routine experimentation, taking as a starting point the physiological conditions under which the compound and target will be expected to interact *in vivo*. From these initial conditions, one may vary the solvent or carrier to aid in solubilizing hydrophobic targets and/or compounds, and may generally increase temperature, pH, and ionic strength in order to make the assay more stringent. One may begin with about 500 pmol of receptor, and 500 pmol of each compound (about 1 µM for the mixture as a whole), with or without excess competitive ligand.

Additionally, one may derive structure activity relationships (SAR) by varying the ratio of compound to target. For example, at a ratio of 1:1 compound:target, only the tightest binding compounds will be selected. However, if the ratio is reduced to 0.3:1 compound:target, compounds with lower affinity will also be selected. A comparison of the MS spectra obtained at different ratios thus provides an indication of the relative affinities of the compounds present in the mixture. Compounds which are present in spectra taken at high ratios of compound to target exhibit a high affinity, while compounds present only in spectra taken at low compound:target ratios exhibit an affinity lower than

the first group. Taken with knowledge of the compounds selected, this information permits one to rapidly determine which features of the compounds (*e.g.*, presence of hydrophilic or hydrophobic groups, hydrogen bonding, aromaticity) are important for activity. The determination is preferably performed at three or more different ratios of compound to

5   target, for example at ratios of 3:1, 1:1, 0.3:1, 0.01:1, and 0.03:1 compound:target.

C. Examples
The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.
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Example 1
(Selection of sUPAR-binding compounds)
Human urokinase plasminogen activator receptor (UPAR) was expressed in a soluble, truncated form as described by Rosenberg *et al.*, WO94/28145, incorporated

15   herein by reference. Peptoid compounds were prepared as described by Bartlett *et al.*, WO91/19735, incorporated herein by reference. Four N-substituted glycine peptoids were prepared, having tyramine in the first (N-terminal) position, 5-aminoindan in the second position, and the following side chains in the third (last) position: diphenyl, phenylphenylether, indan, and 1,4-benzodioxane. The compounds having diphenyl or

20   phenylphenyl ether in the third position were shown to demonstrate affinity for uPAR (> 10 μM), while the other two compounds demonstrated insignificant activity. The ligand uPA$_{1-48}$ was prepared as described in Rosenberg, *supra*.

A mixture of 576 N-substituted glycine peptoids was prepared, including the four compounds specified above. The mixture (1 μM per compound) was contacted with

25   sUPAR (1 μM) in the presence and absence of uPA$_{1-48}$ (2 μM), incubated for 30 minutes, and applied to a SEC HPLC column (Pharmacia HSIO/IO). The compound-target complex eluting in the excluded volume of the column was collected and analyzed on an ES MS (PE-Sciex). The results indicated that the same two peptoids (having diphenyl or phenylphenyl ether in the third position) were selected when contacted in the absence of

30   uPA$_{1-48}$: specificity was confirmed by competition in the presence of uPA$_{1-48}$. Identity of

the compounds was confirmed by fragmentation in CCID mass spec (collision induced dissociation).

The results demonstrate that even compounds having a relatively low affinity for the target may be quickly and accurately selected without the need for

5   deconvolution.

WHAT IS CLAIMED:

1.) A method for selecting a compound capable of binding a selected target, wherein said compound is present within a mixture of similar compounds, said method comprising:

    a) providing a mixture of compounds and a target moiety;

    b) contacting said target with said mixture of compounds to form compound-target complexes;

    c) separating compounds which do not bind to the target from the compound-target complexes; and

    d) passing said compound-target complexes through a mass spectrometer.

2.) The method of claim 1, wherein said mixture of compounds and said target are soluble.

3.) The method of claim 2, wherein said step c) comprises applying said compounds and complexes to a chromatographic column.

4.) The method of claim 3, wherein said chromatographic column comprises a sizing column.

5.) The method of claim 3, wherein said chromatographic column comprises a reversed-phase high performance liquid chromatography column.

6.) The method of claim 1, wherein said mass spectroscope is an electrospray MS.

7.) The method of claim 1, further comprising:

    e) identifying a compound which participated in a compound-target complex.

8.) The method of claim 1, wherein said mixture of compounds comprises a mixture of at least 10 similar compounds.

9.) The method of claim 8, wherein said mixture of compounds comprises a mixture of at least 100 similar compounds.

' 10.) The method of claim 9, wherein said mixture of compounds comprises a mixture of at least 1,000 similar compounds.

11.) A method for determining the relative affinities of two compounds with respect to a target compound, said method comprising:

   a) providing a mixture of compounds and a target moiety;

   b) contacting said target with said mixture of compounds at a first compound:target ratio to form compound-target complexes;

   c) separating compounds which do not bind to the target from the compound-target complexes;

   d) passing said compound-target complexes through a mass spectrometer to obtain a first spectrum; and

   e) repeating steps a) through d) at a second compound:target ratio different from said first compound:target ratio to obtain a second spectrum.

12.) The method of claim 11, wherein said mixture of compounds comprises a mixture of at least 10 similar compounds.

13.) The method of claim 12, wherein said mixture of compounds comprises a mixture of at least 100 similar compounds.

14.) The method of claim 13, wherein said mixture of compounds comprises a mixture of at least 1,000 similar compounds.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N33/53   G01N33/566   H01J49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,95 04160 (ISIS INNOVATION ;SOUTHERN EDWIN (GB); CUMMINS WILLIAM JONATHAN (GB) 9 February 1995	1,2,7
Y	see abstract; claims 14-26	6,8-14
Y	WO,A,94 06017 (SELECTIDE CORP) 17 March 1994 see page 1 - page 4, line 17	6,8-14
Y	WO,A,91 19735 (BARTLETT PAUL A ;SANTI DANIEL V (US); SIMON REYNA J (US)) 26 December 1991 cited in the application see abstract; claims 16-20	8-14
P,A	WO,A,95 25737 (PENN STATE RES FOUND ;BENKOVIC STEPHEN J (US); WINOGRAD NICHOLAS () 28 September 1995	

| | Further documents are listed in the continuation of box C. | [X] | Patent family members are listed in annex. |

Date of the actual completion of the international search	Date of mailing of the international search report
8 May 1996	30.05.96

Authorized officer
Griffith, G

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